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Data supporting use of a centrifugal analyzer such as the COBAS-FARA to study the reduction of methemoglobin with ascorbic acid. This assay system could also be used to investigate the kinetics of other biological systems.

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Kinetic Study of the Reduction of Methemoglobin with Ascorbate Using a COBAS-FARA Centrifugal Analyzer

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Iron(III) hemoglobin (methemoglobin) can be reduced by a number of methods, both enzymic and chemical (1-3), but all of the methods produce unwanted by-products. Recently, we showed that hydrogen gas in the presence of a heterogeneous catalyst can be used to regenerate functional hemoglobin and that the by-product (H^+) can be buffered (3,4).

Since 1942 ascorbic acid has been recognized as a mild reducing agent for methemoglobin (5), although optimizing its use for regeneration of Iron(II) hemoglobin requires the removal of deleterious by-products by dialysis or chromatography (6-8). We have demonstrated that erythrocytes reduce extracellular methemoglobin by utilizing ascorbic acid as an electron shuttling-agent (2). Since ascorbate is a potentially useful reducing agent to regenerate hemoglobin, we used a COBAS-FARA (Roche

Instruments) instrument with a modified program to investigate the reduction kinetics based on initial-rate data.

Stroma-free hemoglobin was obtained from the Letterman Army Institute of Research hemoglobin production facility. It was oxidized with a 20% excess of ferricyanide and dialyzed against distilled water to remove hexacyanoferrate ions. The kinetic protocol was as follows: Ascorbic acid stock solutions were freshly prepared (0.075 - 1.2 M) and placed into sample cups. The instrument was programmed to pipet 5 µL of each ascorbic acid solution into a cuvette with 150 µL of 0.2 M phosphate buffer, pH 7.0. The cuvettes were spun to ensure mixing. A 50 µL aliquot of stock hemoglobin solution was added to each cuvette and the reactions were initiated by spinning the rotor. A_{630} readings were taken at 5 s intervals for 3 minutes. The final reaction mixtures in the cuvettes contained 1.8 mM heme, 1.25 - 20 mM ascorbic acid, and 0.1 M phosphate buffer in a final volume of 300 µL (including water diluents associated with the automatic pipetting system).

Fig. 1 shows a typical plot of the kinetics (initial rate data) of the reduction of 1.8 mM of 100% methemoglobin by 20 mM ascorbate. As can be seen, a straight line was observed over the short time interval studied. Plots for the lower concentrations of ascorbic acid were similar to Fig. 1, except that the data were more scattered. The short reaction time (3 min) was chosen to minimize back reaction (reoxidation) and possible side reactions with oxygen.

Fig. 2 is a plot of the initial rate of methemoglobin reduction vs. ascorbate concentration. The plot is a straight line indicating that the reaction is first order in ascorbate. The order of the reaction was

$d[MetHb]/dt = k[Heme]^1[Ascorbate]^1$. These results can only be explained as a reduction of heme via a concomitant one-electron oxidation of the ascorbate to the relatively stable ascorbate radical, which apparently does not afford further reduction (9).

Presumably our results represent a reduction of the β-chains of hemoglobin because they are preferentially reduced by ascorbic acid (6,7). We also investigated the reduction of hemoglobin/methemoglobin mixtures. This system was complicated by the presence of oxyhemoglobin that produced side reactions (5,8,10). We found that the rates of reduction of 42% methemoglobin samples (1.8 mM total heme) were always about 10-fold lower than those observed for the corresponding 100% methemoglobin samples. Nevertheless, we observed linear initial rates for the reductions with a direct first order dependence on ascorbate concentration. By-products such as hydroxyl radicals (from the reaction of oxyhemoglobin with ascorbate) appear to reduce the reaction rate significantly (8). The most pronounced effects on our initial rates were observed at the lowest ascorbate concentrations.

The data clearly show that a centrifugal analyzer such as the COBAS-FARA may be used to study the reduction of methemoglobin with ascorbic acid. The kinetic data can be obtained quickly and accurately, and this assay system could readily be used to investigate the kinetics of other biological systems.

REFERENCES

1. Bunn, H. F., and Forget, B. G. (1986) *in* Hemoglobin: Molecular, *Genetic and Clinical Aspects*, p. 644, Saunders, Philadelphia.

2. McGown, E. L., Lyons, M. F., Marini, M. A., and Zegna, A. (1990) *Biochim. Biophys. Acta* **1036**, 202-206.

3. McGown, E. L., Dill, K., O'Connor, R. J., Khan, M., LeTellier, Y. C., and Vandegriff, K. D. (1992) *Anal. Biochem.* **207**, 85-89.

4. McGown, E. L., Dill, K., and Khan, M. (1993) *Methods Enzymol.*, in press.

5. Vestling, C. S. (1942) *J. Biol. Chem.* **143**, 439-446.

6. Tomoda, A., Takeshita, M., and Yoneyama, Y. (1978) *J. Biol. Chem.* **253**, 7415-1419.

7. Tomoda, A., Tsuji, A., Matsukawa, S., Takeshita, M., and Yoneyama, Y. (1978) *J. Biol. Chem.* **253**, 7420-7423.

8. Benatti, U., Morelli, A., Guida, L., and Flora, A. (1983) *Biochem. Biophys. Res. Commun.* **111**, 980-987.

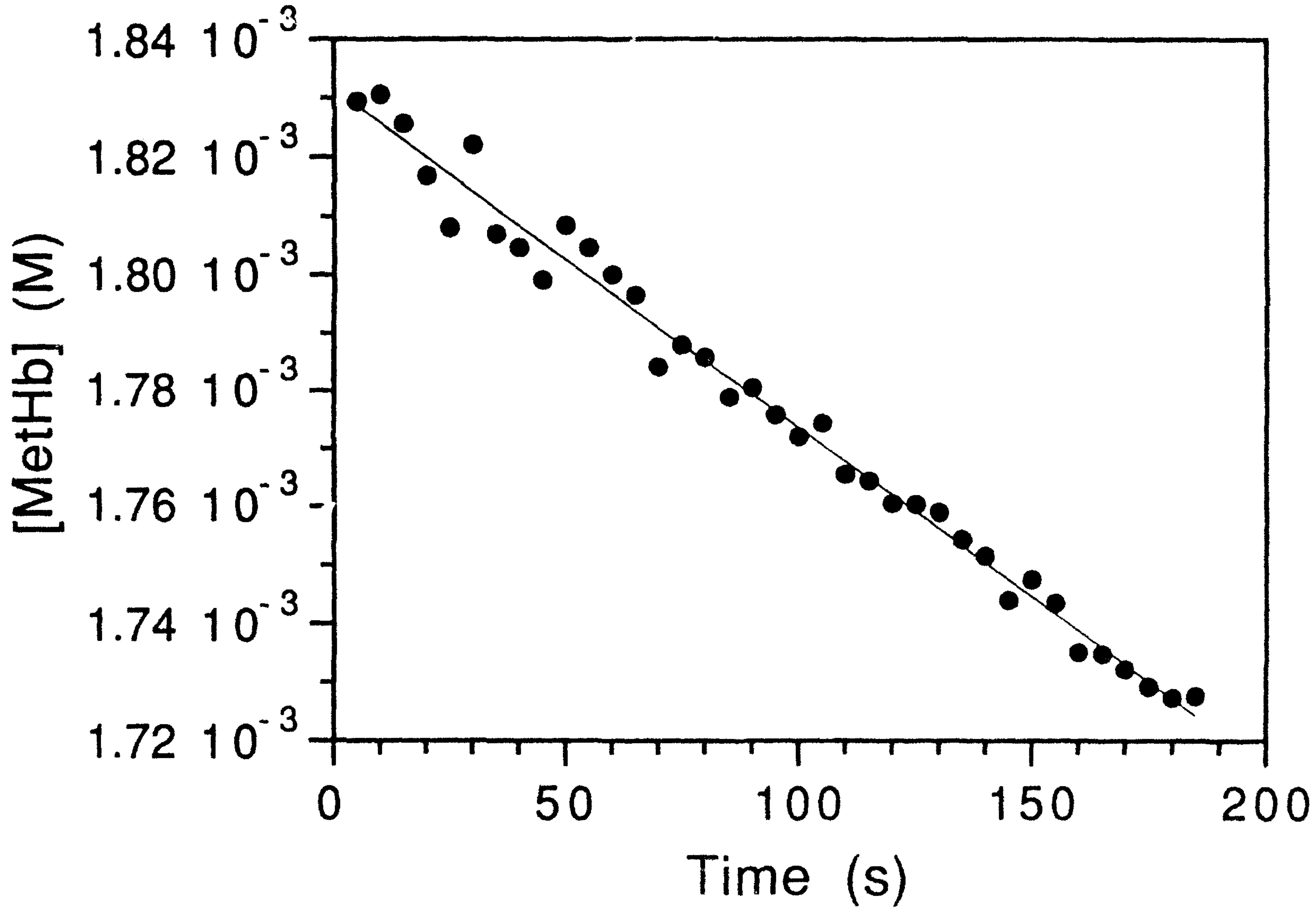
9. Iyanagi, T., Yamazaki, I., and Anan, F. F. (1985) *Biochim. Biophys. Acta* **806**, 255-261.

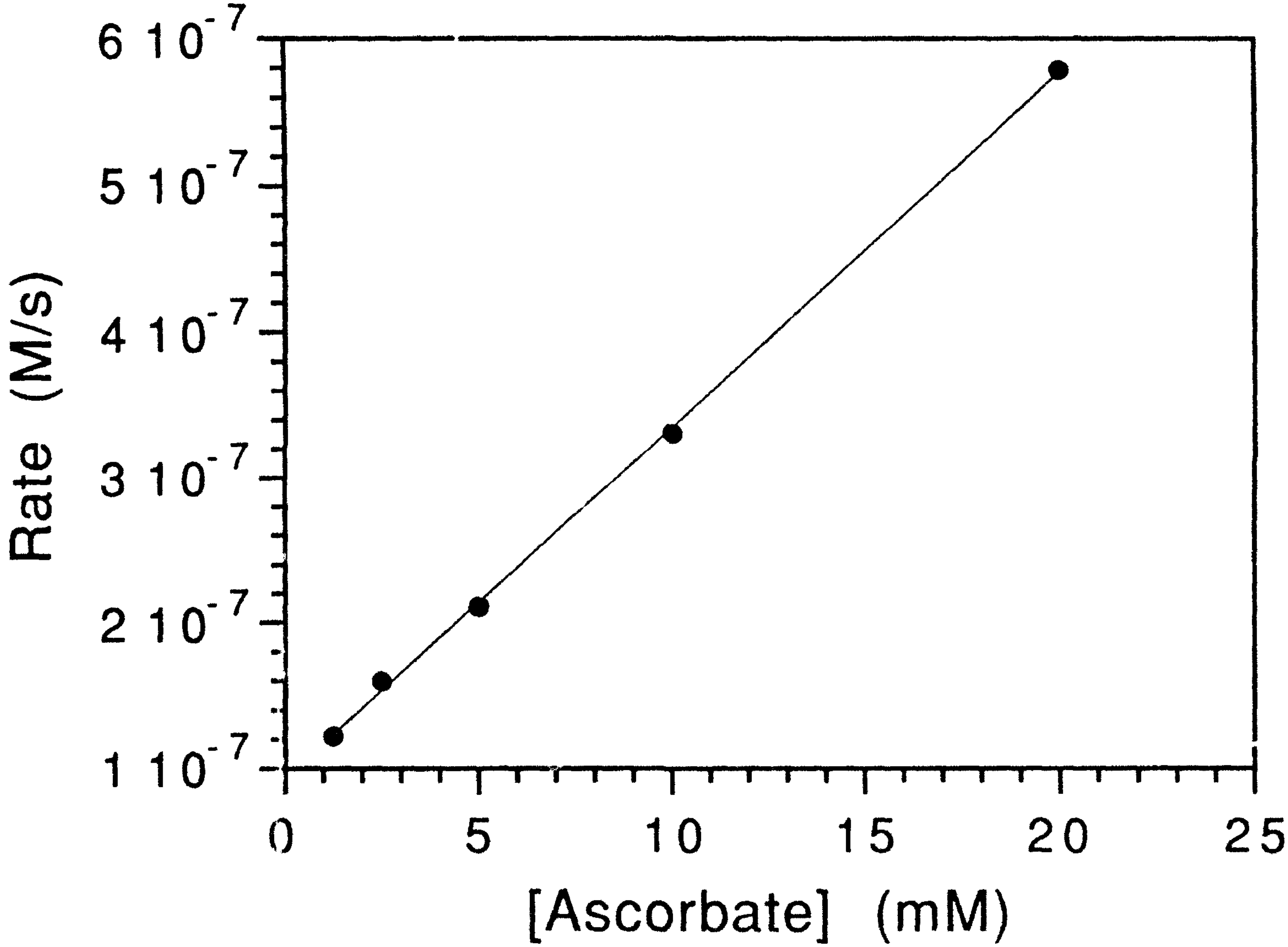
10. Lemberg, R., Legge, J. W., and Lockwood, W. H. (1941) *Biochem. J.* **33**, 754-758.

FIGURE LEGENDS

FIG. 1. Kinetics of the reduction of ~1.8 mM methemoglobin (100% Met) with 20 mM ascorbate. Optical readings were taken every 5 seconds. Each data point represents the average of two data sets.

FIG. 2. Plot of the rate of Methemoglobin reduction as a function of ascorbate. Data were taken from 100% MetHb data and the negative rates (for the reduction of MetHb) are redefined as positive values.


1.84 10-3
1.82 10-3
1.80 10-3
1.78 10-3
1.76 10-3
1.74 10-3
1.72 10-3
[MetHb] (M)
0
50
100
150
200
Time (s)



6 10-7
5 10-7
4 10-7
3 10-7
2 10-7
1 10-7
Rate (M/s)
0
5
10
15
20
25
[Ascorbate] (mM)